Title: **CHRONIC ALGAL (SELENASTRUM CAPRICORNUTUM) TOXICITY TEST**

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1.0 OBJECTIVE

This method measures the chronic toxicity of chemicals or effluents to the fresh water alga, *Selenastrum capricornutum*, during a four-day, static exposure. The endpoint measured is cell growth. This protocol is adapted from ASTM, 1996, Vol. 11.05, pages 29-33.

2.0 HEALTH AND SAFETY

Personnel should wear lab coats, safety goggles and chemical resistant gloves when preparing chemical stocks, and when dosing with test chemicals or effluents.

3.0 PERSONNEL/TRAINING/RESPONSIBILITIES

This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

4.0 REQUIRED AND RECOMMENDED MATERIALS

Environmental chamber
Analytical balance
Light meter
Volumetric pipets
Pipet bulbs
UV-VIS spectrophotometer
Microscope
Mechanical shaker
Volumetric flasks
Serological pipets
pH meter
Thermometer
Media bottles

Counting chambers
Culture flasks & test tubes with caps

5.0 PROCEDURE

5.1 Algal Culturing

5.1.1 Culture Medium

The culture medium is used to maintain the stock cultures of the test organisms, for the controls in each test, and as a diluent in tests.

- Prepare five stock nutrient solutions using reagent grade chemicals as described in Table 1.

- Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of distilled or de-ionized water. Mix well after the addition of each solution. Dilute to 1 L. The final concentration of nutrients in the culture medium is given in Table 2.

- Sterilized by autoclaving. After autoclaving, adjust the pH to 7.5 " 0.1, using 0.1 N sodium/hydroxide or hydrochloric acid, as appropriate.

- Unused sterile medium may be stored at 4 EC for approximately 1 week.

5.1.2 Algal Stocks

Test organisms- *Selenastrum capricornutum*, a unicellular coccoid green fresh-water alga.

- Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically* transferring 1 mL to a culture flask containing control algal culture medium (prepared as described above). The volume of stock culture medium initially prepared will depend on the number of test flasks or tubes to be inoculated later from the stock, or other planned uses. The remainder of the starter culture can be held in reserve for up to six months in the dark at 4 EC.

- Maintain the stock cultures at 25 " 2 EC, under continuous "cool-white" fluorescent lighting of 86 " 8.6 $\mu E/m^2/s$, or 400 " 40 ft-c. Shake continuously at 100 cpm or once daily by hand.

- Transfer stock cultures approximately weekly to new culture medium to maintain a continuous supply of "healthy" cells for tests.

*Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination. Aseptic techniques involve working under a laminar flow hood, wearing gloves, using an alcohol lamp and frequently disinfecting the surface area. Personnel should be trained in these techniques before working with the algal cultures.

5.2 Growth Measurements

5.2.1 Microscope Counts

- Cells counts are determined using a hemocytometer and the light microscope. A total magnification of 100 is sufficient for *S. capricornutum*.

- Both sides of the hemocytometer are counted. 400 cells or all 18 grids (whichever comes first) are counted. Two slides are counted per tube. Clean the hemocytometer with acetone between each sample.

- Cell density is calculated as:
  cells/mL = (number of cells counted / number of grids counted) * 10,000.

5.2.2 Turbidity (Absorbance)

A spectrophotometer is used to determine the turbidity, or absorbance, of the cultures at a wavelength of 664 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, it is useful to construct a calibration curve to establish the relationship between absorbance and cell density.

- Turn on the spectrophotometer and allow the lamp to warm up for approximately 10 minutes.

- Set the wavelength to 664 nm and zero the instrument using a tube containing algal culture medium of the same batch used in the test (no algae).

- Vortex each tube and clean the outside with a Kim-Wipe before reading the absorbance.

5.3 Testing

5.3.1 Test Concentrations

- Range finding tests using a series of test chemical concentrations should be tested initially.

- After a range finding test, a definitive, narrow range of test chemical concentrations are performed to delineate the dose-response curve.

5.3.2 Effluents and Surface Waters

- Prior to testing, surface water and effluent samples should be filtered through a 0.2 µm filter to remove most organisms and particles. Tests should begin as soon as possible, preferably within 24 h of sample collection.

- The selection of effluent test concentrations should be based on the objectives of the study and knowledge of the effluent=s toxicity. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. Surface water samples are usually not diluted in toxicity testing.

- Surface waters and effluents may be toxic and/or nutrient poor. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock solution (except EDTA) is added per liter of surface water/effluent prior to use in preparing test dilutions. Thus all test treatments and controls will contain at least the basic amount of nutrients.

5.3.3 Start of the Test

- Prepare secondary chemical stocks using sterile algal culture media. Prepare the test concentrations, using sterile algal culture media as diluent, with a final volume of 25 mL in each test tube. Prepare three replicate tubes per treatment. Remember to keep the concentration of carrier solvents constant in the treatments and controls. Acetone should be kept at 0.1%. It may be desirable to confirm the chemical concentration of the stock analytically. Preparation of chemical stocks and test concentrations should be performed in a fume hood.

- Determine the density of cells (cells/mL) in the stock culture (4-7 days old). Calculate the volume of inoculum needed to yield an initial cell density of 20,000 cells/mL in the test tubes (25 mL final volume).

- The test begins when the algae are added to the test tubes.

- Count the tubes immediately after inoculation to determine the cell density in each vessel at time “zero”.

- Test tubes are incubated under the culture conditions described above. Tubes should be randomly rotated in their racks each day to minimize possible spatial differences in illumination and temperature on growth rate.

- Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one to two days in the test. It may be desirable, therefore, to determine the algal growth response daily.

5.3.4 End of the Test

- The test is terminated 96 h after initiation. The algal growth in each tube is measured by direct cell counts and turbidity (light absorbance). Changes in cell size or shape should also be noted.

- The data are then analyzed using appropriate statistics and an EC_{50} is calculated.

- Control test tubes are emptied into the sink. Treatment test tubes must be emptied into appropriate spent chemical containers and immediately solvent rinsed. See glassware washing SOP.

6.0 QUALITY ASSURANCE/QUALITY CONTROL

Personnel should follow good laboratory practices during algal culturing and testing. The minimum number of replicates tested should be three. The standard error associated with the mean of the measurements for each treatment should be = 30%.

7.0 REFERENCES

ASTM (American Society for Testing and Materials) (1996) Annual Book of ASTM Standards. Vol. 11.05, ASTM, West Conshohocken, PA 1402 pp

8.0 TABLES

8.1 Nutrient Stock Solutions for Maintaining Algal Stock Cultures and Test Control Cultures

Nutrient Stock Solution	Compound	Amount dissolved in 500 mL de-ionized H_2O
1	$MgCl_2 \cdot 6H_2O$	6.08g
	$CaCl_2 \cdot 2H_2O$	2.20g
	H_3BO_3	92.8mg
	$MnCl_2 \cdot 4H_2O$	208.0mg
	$ZnCl_2$	1.64mg*
	$FeCl_3 \cdot 6H_2O$	79.9mg
	$CoCl_2 \cdot 6H_2O$	0.714mg*
	$Na_2MoO_4 \cdot 2H_2O$	3.63mg*
	$CuCl_2 \cdot 2H_2O$	0.006mg*
	$Na_2EDTA \cdot 2H_2O$	150.0mg
2	$NaNO_3$	12.750g
3	$MgSO_4 \cdot 7H_2O$	7.350g
4	K_2HPO_4	0.522g
5	$NaHCO_3$	7.50g

* $ZnCl_2$ -Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

* $CoCl_2 \cdot 6H_2O$ -Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution

to Stock #1.

* $Na_2MoO_4 \cdot 2H_2O$ -Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #1.

* $CuCl_2 \cdot 2H_2O$ -Weigh out 60 mg and dilute to 1000 mL. Add 1 mL of this solution to Stock #1.

8.2 Final Concentration of Nutrients in the Culture Medium.

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	22.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14
Micronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$Na_2EDTA \cdot 2H_2O$	300		